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TITLE: Fertile transgenic Zea mays plant comprising heterologous DNA encoding Bacillus thuringiensis endotoxin

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INVENTOR-INFORMATION:

| NAME | CITY | STATE | ZIP CODE | COUNTRY |
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US-CL-CURRENT: 800/302; 536/23.71

ABSTRACT:

Fertile transgenic Zea mays (corn) plants which stably express heterologous DNA which is heritable are provided along with a process for producing said plants. The preferred process comprises the microprojectile bombardment of friable embryogenic callus from the plant to be transformed. The process may be applicable to other graminaceous cereal plants which have not proven stably transformable by other techniques.

6 Claims, 8 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 6

CLAIMS:

What is claimed is:

1. A fertile transgenic Zea mays plant of the R0 generation containing heterologous DNA encoding Bacillus thuringiensis endotoxin, wherein said DNA is expressed so that the plant exhibits resistance to an insect, wherein said expression is not present in said plant not containing said DNA, and wherein said DNA is transmitted through a complete normal sexual cycle of the R0 plant to the R1 generation, and wherein said DNA is introduced into said plant by microprojectile bombardment of Zea mays callus cells.

2. The transgenic plant of claim 1 wherein said DNA comprises a promoter.

3. The transgenic plant of claim 1 which is selected from the group consisting of field corn, popcorn, sweet corn, flint corn and dent corn.

4. A seed produced by the transgenic plant of claim 1 which comprises a replication of said heterologous DNA.

5. An R1 transgenic Zea mays plant derived from the plant of claim 1 wherein said R1 plant expresses said heterologous DNA so that the R1 plant exhibits said phenotypic characteristics.

6. A progeny transgenic Zea mays plant derived from the plant of claim 5 wherein said progeny plant expresses said heterologous DNA so that the progeny plant exhibits said phenotypic characteristics.

observed for DNA from control callus in any of the above treatments.

These results demonstrate that the HPT coding sequence is not present in PH1 callus as intact pHYGI1 or as a small non-chromosomal plasmid. They are consistent with incorporation of the hygromycin gene into high molecular weight DNA. Further, Southern blot analyses demonstrated that the HPT coding sequence is contiguous with the 35S promoter sequence.

PH1 callus was transferred from all of the concentrations of hygromycin used in the inhibition study to RM5 medium and plants were regenerated as described in Example I. A total of 65 plants were produced from PH1 and a total of 30 plants were produced from control callus.

To demonstrate that the introduced DNA had been retained in the R0 tissue, a Southern blot was performed as previously described on BamHI digested leaf DNA from three randomly chosen R0 plants of PH1. The blot was probed with the HPT probe as in Example I. As shown in FIG. 4, a 1.05 Kb band was observed with all three plants indicating that the HPT coding sequence was present. No band was observed for DNA from a control plant.

Controlled pollinations of mature PH1 plants were conducted by standard techniques with inbred *Zea mays* lines A188, B73, and Oh43. Seed was harvested 45 days post-pollination and allowed to dry further 1–2 weeks.

The presence of the hygromycin resistance trait in the R1 progeny was evaluated by the root elongation bioassay, an etiolated leaf bioassay, and by Southern blotting. Two ears each from regenerated PH1 and control plants were selected for analysis. The pollen donor was inbred line A188 for all ears. The results are shown in FIG. 5 and in Table 9, below.

TABLE 9

| | ANALYSIS OF PH1 R1 PLANTS | | | | | | |
|---|---|---|---|---|---|---|---|
| PH1 PLANT | ROOT ASSAY | LEAF ASSAY | BLOT | CONT PLANT | ROOT ASSAY | LEAF ASSAY | BLOT |
| 3.1 | + | ND | + | 4.1 | – | ND | ND |
| 3.2 | – | ND | – | 4.2 | – | ND | ND |
| 3.3 | – | ND | – | 4.3 | – | ND | ND |
| 3.4 | – | ND | – | 4.4 | – | ND | ND |
| 3.5 | – | ND | – | 4.5 | – | ND | ND |
| 3.6 | + | ND | + | 4.6 | – | ND | ND |
| 3.7 | – | ND | – | 4.7 | – | ND | ND |
| | | | | 2.1 | – | ND | – |
| 10.1 | + | + | + | 1.1 | – | – | – |
| 10.2 | + | + | + | 1.2 | – | – | ND |
| 10.3 | – | | ND | 1.3 | – | – | ND |
| 10.4 | – | – | – | 1.4 | – | – | ND |
| 10.5 | – | – | – | 1.5 | – | – | ND |
| 10.6 | – | – | – | 1.6 | – | – | ND |
| 10.7 | – | – | – | 1.7 | – | – | ND |
| 10.8 | ND | + | + | 1.8 | – | – | ND |

Key: + = transgenic; – = nontransgenic; ND = not done

The presence of the HPT gene was confirmed in two R2 progeny deriving from a PH1 maternal parent.

Plant PH1.3.1 (see Table 9) was pollinated with Oh43 pollen. Nine seeds derived from this cross were germinated, DNA was prepared from the leaves of four of the progeny plants, and analyzed by Southern blotting using the HPT coding sequence probe. Two of the four plants tested contained the HPT sequence in high copy number. Although the HPT gene was present in two of the plants, expression of the gene could not be detected in the etiolated leaf assay.

Plant PH1.10.8 (Table 9) was used to pollinate B73 and was also selfed. Fifty of the progeny from the outcross were tested for HPT expression in both root and leaf assays. No expression was detected. Likewise Southern blots on DNA from eight of the progeny did not detect the presence of the HPT gene. In the progeny from the self cross, no evidence for the presence of the gene was obtained in leaf assays of nine progeny, or from Southern blots of the DNA from four of these plants.

The recombinant DNA was only shown to be inherited by progeny when a transgenic plant (both R0 and R1) was used as a female. This is suggestive of maternal inheritance of the recombinant DNA for PH1.

All of the publications and patent documents cited hereinabove are incorporated by reference herein. The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

What is claimed is:

**1**. A fertile transgenic *Zea mays* plant containing an isolated preselected DNA construct comprising a promoter and encoding a *Zea mays* seed storage protein under the control or said promoter, wherein the DNA construct is expressed as said seed storage protein so that the level or a seed storage protein amino acid in the seeds of said transgenic plant is substantially increased above the level in the seeds of a *Zea mays* plant which only differ from the seeds of said transgenic *Zea mays* plant in that said DNA construct is absent and wherein said DNA construct is transmitted through a complete normal sexual cycle of the transgenic plant to the next generation.

**2**. The plant of claim **1** selected from the group consisting of field corn, popcorn, sweet corn, flint corn, and dent corn.

**3**. The plant of claim **1** wherein said preselected DNA construct further comprises and expresses a selectable marker gene or a reporter gene.

**4**. The plant of claim **3** wherein the selectable marker gene confers resistance or tolerance to a compound selected from the group consisting of hygromycin, kanamycin, G418, 2,2-dichloropropionic acid and neomycin.

**5**. The plant of claim **4** wherein the selectable marker gene confers resistance or tolerance to hygromycin.

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6. The plant of claim 3 wherein the preselected DNA construct further comprises and expresses a reporter gene.

7. The seed produced by the plant of claims 1 or 3 which has inherited the preselected DNA construct.

8. The transgenic *Zea mays* plant of claim 1, wherein the seed storage protein is the 10-kD zein protein, which is expressed so that the level of the whole kernel methionine is substantially increased above the whole kernel methionine level in the corresponding *Zea mays* plant which only differs from said transgenic *Zea mays* plant in that said DNA construct is absent.

9. The transgenic *Zea mays* plant of claim 1 wherein the preselected DNA construct comprises less than about 30 kilobases.

10. The transgenic *Zea mays* plant of claims 1 or 8 wherein the preselected DNA construct is introduced into cells of the plant by microprojectile bombardment.

11. The transgenic *Zea mays* plant of claims 1 or 8 wherein the promoter is a promoter isolated from *Zea mays*.

12. The transgenic *Zea mays* plant of claim 1 or 8 wherein the DNA construct is chimeric.

13. The transgenic *Zea mays* plant of claim 1 wherein the amino acid is methionine.

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-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCTTACCTAC TAATTGTTCT TGG 23

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CAGGGTACAT ATTTGCCTTG GG 22

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AACCCTGAAT GGAAGTGC 18

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ACGGACAGAT GCAGATTGG 19

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Pro Arg Gly Ser Thr
1 5

What is claimed is:

**1**. A fertile transgenic *Zea mays* plant comprising an isolated heterologous chimeric DNA comprising a DNA sequence having SEQ ID NO:5, wherein said heterologous chimeric DNA is expressed so that the plant exhibits resistance to an insect, wherein said expression is not present in said plant not containing said heterologous chimeric DNA, and wherein said heterologous chimeric DNA is transmitted through a complete normal sexual cycle of the transgenic plant to the progeny generation.

**2**. The transgenic *Zea mays* plant of claim **1** wherein the heterologous chimeric DNA comprises a promoter.

**3**. The transgenic *Zea mays* plant of claim **1** wherein the heterologous chimeric DNA encodes a chloroplast transit peptide.

**4**. The transgenic *Zea mays* plant of claim **1** wherein the heterologous chimeric DNA comprises a selectable marker gene or a reporter gene.

**5**. The transgenic *Zea mays* plant of claim **4** wherein the selectable marker gene confers resistance or tolerance to a compound selected from the group consisting of glyphosate, bromoxynil, imidazolinone, sulfonylurea, methotrexate, 5-methyl typtophan, kanamycin, G418, 2,2-dichloropropionic acid, phosphinothricin, and neomycin.

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6. A seed produced by the transgenic plant of claim 1 which comprises said heterologous chimeric DNA.

7. A progeny *Zea mays* plant derived from the transgenic plant of claim 1 wherein said progeny plant expresses said heterologous chimeric DNA so that the progeny plant exhibits said insect resistance.

8. A seed derived from the progeny plant of claim 7 wherein said seed comprises said heterologous chimeric DNA.

9. The transgenic plant of claim 1 wherein the plant is obtained by a process comprising the steps of:

(i) bombarding intact regenerable *Zea mays* cells with microprojectiles coated with said heterologous chimeric DNA;

(ii) identifying or selecting a population of transformed cells; and

(iii) regenerating a fertile transgenic plant therefrom.

10. A fertile transgenic *Zea mays* plant comprising an isolated heterologous chimeric DNA comprising a DNA sequence having SEQ ID NO:5 operably linked to an actin promoter, wherein said heterologous chimeric DNA is expressed so that the plant exhibits resistance to an insect, wherein said expression is not present in said plant not containing said heterologous chimeric DNA, and wherein said heterologous chimeric DNA is transmitted through a complete normal sexual cycle of the transgenic plant to the progeny.

11. A fertile transgenic *Zea mays* plant comprising an isolated heterologous chimeric DNA comprising a DNA sequence having SEQ ID NO:5 operably linked to a histone promoter, wherein said heterologous chimeric DNA is expressed so that the plant exhibits resistance to an insect, wherein said expression is not present in said plant not containing said heterologous chimeric DNA, and wherein said heterologous chimeric DNA is transmitted through a complete normal sexual cycle of the transgenic plant to the progeny.

12. The transgenic *Zea mays* plant of claim 11 wherein the histone promoter is a maize histone promoter.

13. The transgenic *Zea mays* plant of claim 10 or 11 wherein the heterologous chimeric DNA encodes a chloroplast transit peptide.

14. The transgenic *Zea mays* plant of claim 10 or 11 wherein the heterologous chimeric DNA comprises a selectable marker gene or a reporter gene.

15. The transgenic *Zea mays* plant of claim 14 wherein the selectable marker gene confers resistance or tolerance to a compound selected from the group consisting of glyphosate, bromoxynil, imidazolinone, sulfonylurea, methotrexate, 5-methyl tryptophan, kanamycin, G418, 2,2-dichloropropionic acid, phosphinothricin, and neomycin.

16. A seed produced by the transgenic plant of claim 10 or 11 which comprises said heterologous chimeric DNA.

17. A progeny *Zea mays* plant derived from the transgenic plant of claim 10 or 11 wherein said progeny plant expresses said heterologous chimeric DNA so that the progeny plant exhibits said insect resistance.

18. A seed derived from the progeny plant of claim 17 wherein said seed comprises said heterologous chimeric DNA.

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The presence of the HPT gene was confirmed in two R2 progeny deriving from a PH1 maternal parent.

Plant PH1.3.1 (see Table 9) was pollinated with Oh43 pollen. Nine seeds derived from this cross were germinated, DNA was prepared from the leaves of four of the progeny plants, and analyzed by Southern blotting using the HPT coding sequence probe. Two of the four plants tested contained the HPT sequence in high copy number. Although the HPT gene was present in two of the plants, expression of the gene could not be detected in the etiolated leaf assay.

Plant PH1.10.8 (Table 9) was used to pollinate B73 and was also selfed. Fifty of the progeny from the out-cross were tested for HPT expression in both root and leaf assays. No expression was detected. Likewise Southern blots on DNA from eight of the progeny did not detect the presence of the HPT gene. In the progeny from the self cross, no evidence for the presence of the gene was obtained in leaf assays of nine progeny, or from Southern blots of the DNA from four of these plants.

The recombinant DNA was only shoe to be inherited by progeny when a transgenic plant (both R0 and R1) was used as a female. This is suggestive of maternal inheritance of the recombinant DNA for PH1.

All of the publications and patent documents cited hereinabove are incorporated by reference herein. The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

What is claimed is:

**1**. A fertile transgenic *Zea mays* plant comprising a preselected DNA sequence encoding a *Bacillus thuringiensis* endotoxin, wherein the preselected DNA sequence is adjusted to be more efficently expressed in maize than the native *B. thuringiensis* DNA sequence encoding said endotoxin, and wherein said preselected DNA is heritable.

**2**. The transgenic *Zea mays* plant of claim **1** wherein the preselected DNA sequence comprises an increased G+C content of the degenerate third base of the codons.

**3**. The transgenic *Zea mays* plant of claim **1** or wherein the preselected DNA sequence comprises a sequence encoding the HD73 endotoxin of *Bacillus thuringiensis*.

**4**. A seed produced by the transgenic *Zea mays* plant claim **1**, **3**, which comprises said preselected DNA sequence.

**5**. The transgenic *Zea mays* plant of claim **1** wherein the preselected DNA sequence encodes a truncated *Bacillus thuringiensis* endotoxin.

**6**. The transgenic *Zea mays* plant of claim **1** wherein the preselected DNA sequence comprises a promoter.

**7**. A fertile inbred transgenic *Zea mays* plant comprising a preselected DNA sequence encoding a *Bacillus thuringiensis* endotoxin, wherein the preselected DNA sequence is adjusted to be more efficiently expressed in maize than the native *B. thuringiensis* DNA sequence encoding said endotoxin and wherein the preselected DNA sequence is heritable.

**8**. A fertile hybrid transgenic *Zea mays* plant comprising a preselected DNA sequence encoding a *Bacillus thuringiensis* endotoxin, wherein the preselected DNA sequence is adjusted to be more efficiently expressed in maize than the native *B. thuringiensis* DNA sequence encoding said endotoxin, and wherein the preselected DNA sequence is heritable.

**9**. A fertile transgenic *Zea mays* plant comprising a preselected heritable DNA sequence encoding a *Bacillus thuringiensis* endotoxin, wherein the preselected DNA sequence is adjusted to be more efficiently expressed in maize than the native *B. thuringiensis* DNA sequence encoding said endotoxin, and wherein the preselected DNA sequence further comprises a selectable marker gene or a reporter gene.

**10**. The transgenic *Zea mays* plant of claim **9** wherein the preselected DNA sequence comprises a sequence encoding the HD73 endotoxin of *Bacillus thuringiensis*.

**11**. The transgenic *Zea mays* plant of claim **9** wherein the preselected DNA sequence comprises a sequence encoding the HD1 endotoxin of *Bacillus thuringiensis*.

**12**. A seed produced by the transgenic *Zea mays* plant of claim **9**, **10** or **11** which comprises said preselected DNA sequence.

**13**. The transgenic *Zea mays* plant of claim **9** wherein the DNA sequence encodes a truncated *Bacillus thuringiensis* endotoxin.

**14**. The transgenic *Zea mays* plant of claim **9** wherein the preselected DNA sequence further comprises a promoter operably linked to said DNA sequence encoding said endotoxin and a promoter operably linked to said selectable marker gene.

**15**. A fertile inbred transgenic *Zea mays* plant comprising a preselected heritable DNA sequence encoding a *Bacillus thuringiensis* endotoxin, wherein the preselected DNA sequence is adjusted to be more efficiently expressed in maize than the native *B. thuringiensis* DNA sequence encoding said endotoxin, and wherein the preselected DNA sequence further comprises a selectable marker gene or a reporter gene.

**16**. A fertile hybrid transgenic *Zea mays* plant comprising a preselected heritable DNA sequence encoding a *Bacillus thuringiensis* endotoxin, wherein the preselected DNA sequence is adjusted to be more efficiently expressed in maize than the native *B. thuringiensis* DNA sequence encoding said endotoxin, and wherein the preselected DNA sequence further comprises a selectable marker gene or a reporter gene.

**17**. The transgenic *Zea mays* plant of claim **9** wherein the selectable marker gene confers resistance or tolerance to a compound selected from the group consisting of hygromycin, sethoxydim, haloxyfop, glyphosate, methotrexate, imidazoline, sulfonylurea, triazolopyrimidine, s-triazine, bromoxynil, phosphinothricin, kanamycin, G418, 2,2-dichloropropionic acid and neomycin.

**18**. The transgenic plant of claim **17** wherein the compound is phosphinothricin.

**19**. The transgenic plant of claim **17** wherein the compound is glyphosate.

**20**. The transgenic plant of claim **17** wherein the compound is kanamycin.

**21**. The transgenic plant of claim **17** wherein the compound is hygromycin.

**22**. The transgenic plant of claim **9** wherein the DNA encoding the *Bacillus thuringiensis* endotoxin is fused in frame with said selectable marker or reporter gene.

**23**. The inbred transgenic plant of claim **15** wherein the DNA encodes a truncated *Bacillus thuringiensis* endotoxin.

**24**. The hybrid transgenic plant of claim **18** wherein the DNA encodes a truncated *Bacillus thuringiensis* endotoxin.

**25**. The transgenic plant of claim **5**, **23** or **24** wherein the truncated *Bacillus thuringiensis* endotoxin comprises about the N-terminal 50% of the endotoxin.

**26**. The transgenic plant of claim **1** or **9** wherein the preselected DNA further encodes a protease inhibitor.

**27**. The transgenic plant of claim **6** or **14** wherein the preselected DNA sequence further comprises the maize AdhIS first intron or the maize Shrunken-2 first intron positioned between the promoter and the DNA encoding said endotoxin.

**28**. The transgenic plant of claim **6** or **14** wherein the preselected DNA sequence further comprises a manopine synthase promoter, a nopaline synthase promoter or an octopine synthase promoter.

**29**. The transgenic plant of claim **6** or **14** wherein the promoter is the CaMV 35S or 19S promoter.

30. A population of plants obtained by breeding the transgenic plants of claim 1 or 14 wherein the preselected DNA sequence is transmitted by Mendelian inheritance through both male and female parent plants.

31. An inbred insect-resistant transgenic *Zea mays* plant prepared by a process comprising:

(a) crossing a fertile transgenic *Zea mays* plant comprising a preselected DNA sequence encoding a *Bacillus thuringiensis* endotoxin, wherein the preselected DNA sequence is adjusted to be more efficiently expressed in maize than the a *B. thuringiensis* DNA sequence encoding said endotoxin, and wherein said DNA sequence is heritable, with a member of a second inbred *Zea mays* line;

(b) recovering insect-resistant transgenic progeny plants from said cross;

(c) back-crossing one of the transgenic progeny plant with a member of said second inbred line;

(d) recovering insect-resistant transgenic progeny plants from said cross; and

(e) repeating steps (b) and (c) to obtain said inbred plant.

32. The inbred transgenic *Zea mays* plant of claim 31 wherein said preselected DNA sequence encodes a truncated *Bacillus thuringiensis* endotoxin.

33. A transgenic insect-resistant hybrid plant prepared by crossing the inbred plant of claim 31 or 32 with an inbred *Zea mays* line, and recovering said hybrid plant.

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